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Regulation of vascular endothelial growth factor by hypoxia and its modulation by the von Hippel-Lindau tumor suppressor gene

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Adaptation to decreased oxygen tension is a fundamental requirement of all living organisms. Two well understood adaptations in higher organisms to a decrease in oxygen delivery to the tissues are an increase in respiratory rate and an increase in red blood cell mass. These physiological adaptations are achieved by specific oxygen sensing cells in the carotid body and kidney, respectively. At a molecular level this adaptation to hypoxia involves the specific regulation of hypoxia responsive genes such as tyrosine hydroxylase and erythropoietin (Epo). New blood vessel formation or angiogenesis is also an adaptive response to cell and tissue hypoxia [1]. Indeed, the increased coronary collateral circulation of the heart seen in coronary artery disease, tumor angiogenesis that is required for tumor growth and metastasis, and diabetic retinopathy are recognized as responses to hypoxia [2–4]. Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is a potent angiogenic factor and endothelial cell-specific mitogen [5, 6] that is regulated by hypoxia *in vitro* and *in vivo* [1]. Furthermore, the neovascular response associated with many tumors as well as experimentally induced retinopathy can be inhibited with neutralizing antiserum to VEGF [4, 7]. VEGF thus appears to be an important mediator of hypoxia induced angiogenesis *in vivo*. This review will describe studies that we and others have performed investigating the mechanism of hypoxic induction of VEGF.

Regulation of VEGF by hypoxia occurs at the level of the steady state mRNA

VEGF mRNA has been demonstrated to be induced by hypoxia 8 to 30-fold in multiple cell lines *in vitro* [1, 8–10]. We have shown that primary neonatal rat cardiac myocytes increase the level of VEGF mRNA 25.0 ± 11.4-fold by 24 hours of exposure to 1% oxygen [9]. A significant increase in VEGF mRNA can be seen as early as three hours after exposure to hypoxia. This increase in mRNA is associated with a commensurate increase in VEGF protein production and release into the tissue culture media [9], arguing against a significant role for translational regulation of VEGF synthesis with hypoxia.

Specific riboprobes that allow for the identification of the 121, 165, 189 and 206 amino acid isoforms of VEGF were developed and revealed marked differences in the steady state levels of the mRNA for these isoforms in different cell lines and tissues. However, we have not observed a significant change in the relative amounts of the different isoforms in response to hypoxia [9].

Transition metals stimulate VEGF production

It has been hypothesized that the ability of the transition metals cobalt and manganese to stimulate Epo production is due to their effect on an oxygen-sensing heme protein whose activity changes depending on the presence or absence of oxygen binding to the heme moiety [11]. Cobalt and manganese can substitute for iron in the protoporphyrin ring, but the resulting metal protoporphyrins bind oxygen either poorly or not at all, thus locking the protein in a deoxy conformation and simulating the deoxy state. We have demonstrated that cobalt and manganese can stimulate VEGF mRNA in multiple cell lines [8, 9]. The stimulation of VEGF by transition metals provides evidence for a common oxygen-sensing system regulating the expression of VEGF and Epo.

Regulation of hypoxic induction of VEGF mRNA by activated second messenger systems

Hypoxia has been shown to increase calcium influx and membrane bound protein kinase C in a variety of cell types. Furthermore, these same second messengers have been shown to stimulate VEGF mRNA [12]. However, only protein kinase C inhibition with H7 (20 μM), as opposed to protein kinase A or calcium calmodulin kinase II inhibition, demonstrated any significant inhibition of VEGF mRNA induction with hypoxia [9]. This suggests that induction of VEGF synthesis may occur through more than one pathway.

Transcriptional rate for VEGF is increased threefold by hypoxia

While the steady state mRNA for VEGF is increased 8- to 30-fold by hypoxia, we and others have found that transcriptional rate for VEGF as assessed by nuclear run off transcription assay is only increased two- to threefold by hypoxia [10, 13]. In order to investigate the *cis*-regulatory sequences that mediate this response to hypoxia, we cloned the rat genomic sequences encoding VEGF. A 2 kb fragment containing VEGF flanking sequence was used in transient transfection assays with the luciferase reporter

gene. A fourfold increase in reporter gene activity was observed when cells were exposed to 1% oxygen [10]. Inclusion of VEGF 5′ untranslated or 3′ untranslated sequences in their native orientations relative to the reporter gene did not result in any significant further increase in the hypoxic induction mediated by the 2 kb promoter fragment in transient transfection assays.

Identification of a functional HIF-1 site in the VEGF 5′ promoter

Multiple constructs containing VEGF 5′ promoter fragments were generated by deletional analysis of the construct described above. Initially a 215 bp fragment was identified that was capable of conferring hypoxia inducibility to the reporter gene. This fragment was functional in this capacity in either orientation relative to a heterologous promoter. Within this fragment a region of 28 bp that were 100% conserved between the rat and human VEGF genes was identified and oligonucleotides corresponding to this sequence were generated [10]. This synthetic site was placed 5′ to the minimal VEGF promoter in transient expression assays and conferred a significant increase in the hypoxia/normoxia ratio of reporter gene activity. Introduction of a three base pair change, specifically in a potential hypoxia-inducible factor 1 (HIF-1) [14] binding site in the 28 bp sequence, resulted in abrogation of this hypoxia inducible response [10].

In electromobility band shift assays, a specific hypoxia inducible DNA band shift was observed with this 28 bp radiolabeled VEGF DNA sequence. This band shift was competitively blocked by an excess of either the unlabeled 28 bp VEGF DNA sequence or by an oligonucleotide containing the HIF-1 binding site from the Epo hypoxia responsive enhancer. Conversely, the mutant oligonucleotide was unable to competitively inhibit the hypoxia inducible band shift with the wild-type sequence, nor was it able to specifically retard a hypoxia-inducible species in the band shift assay.

Other *cis*-elements and *trans*-acting factors are likely to be involved in the hypoxic induction of VEGF in addition to HIF-1. For example the VEGF minimal promoter, which contains 3 SP1 sites, is modestly hypoxia-inducible in the transient transfection reporter assay. SP1 binding is dependent on redox state with a reducing environment (which occurs with hypoxia) stimulating SP1 binding as detected by DNA band shift and reporter gene assays [15]. Similarly, AP-1 which has several consensus cognate binding sites in the VEGF promoter has been identified as a redox-sensitive transcription factor [16]. It is interesting to note that cooperativity between HIF-1 and other transcription factors in the hypoxia inducible increase in the transcription rate has been noted for other hypoxia inducible genes such as lactate dehydrogenase [17] and Epo [18–20].

VEGF mRNA is stabilized by hypoxia

Preliminary studies using cycloheximide [1] as well as the discrepancy between the degree of increase in steady-state VEGF mRNA levels and the results of the nuclear run off studies suggested that post-transcriptional mechanisms were active in the hypoxic induction of VEGF. Actinomycin D chase experiments performed by our lab and others [13, 21–23] have shown a 2.5- to eightfold increase in the half life of VEGF mRNA with hypoxia.

Identification of sequences in the VEGF 3′UTR that mediate the rapid turnover of VEGF mRNA and its stabilization by hypoxia

The 3′ region of the VEGF gene was cloned from both a genomic and cDNA library [10]. The transcription termination site was mapped initially by Northern analysis using a battery of genomic probes and definitively identified by the sequencing of multiple independent cDNA inserts. The most frequently used polyadenylation site was identified 1.9 kb 3′ to the translation termination codon. This 1.9 kb of sequence contains multiple pentameric AUUUA sequence motifs that have previously identified to mediate the rapid turnover of numerous cytokine and oncogene mRNAs [24].

An *in vitro* RNA degradation assay was developed using ^{32}P labeled, capped, polyadenylated VEGF 3′ untranslated region (UTR) transcripts synthesized *in vitro* with SP6 RNA polymerase [23]. These transcripts were incubated with S-100 cytoplasmic extract prepared from hypoxic or normoxic cells. The incubation was terminated at defined time intervals and analyzed on a formaldehyde agarose gel. After transfer to nylon membrane the amount of primary undegraded transcript was quantified by phosphorimaging analysis. Several VEGF 3′ UTR transcripts containing progressive deletions from the 3′ end were assayed in this system. Using only normoxic extracts, two regions of the 3′ UTR were identified that each independently resulted in a twofold increase in VEGF stability when deleted in the *in vitro* assay. These instability sequences co-localized with consensus nonameric instability elements that have been identified as the minimal elements capable of conferring rapid degradation to a heterologous gene, and have been previously identified in multiple cytokine and oncogene 3′ UTRs [25, 26].

When comparing the stability of the full length VEGF 3′ UTR transcript in the RNA degradation assay using normoxic versus hypoxic extracts, we observed a significant increase in stability of the full length transcript with the hypoxic extracts. This preferential stability of the full-length transcript was lost upon progressive deletional analysis of the VEGF 3′ UTR commencing from the 3′ end, thus allowing localization of a region in the VEGF 3′ UTR that is critical for the hypoxic stabilization of VEGF 3′ UTR transcripts in this *in vitro* assay.

Identification of a hypoxia-inducible protein complex which binds to the VEGF mRNA 3′ untranslated region

RNA transcripts of different regions of the VEGF 3′-UTR incubated with S100 extract permitted the identification by electromobility band shift assay (EMSA) of both constitutive and hypoxia-induced VEGF mRNA binding proteins [23]. The constitutive protein complex was found to map between nucleotides 909-1255 (GenBank Accession #U22372) of the VEGF 3′ UTR. A region of this fragment was identified with high homology to a RNA fragment previously identified in the tyrosine hydroxylase 3′ UTR that forms a hypoxia-inducible RNA-protein complex [27]. Furthermore, a 162 bp fragment of the tyrosine hydroxylase 3′ UTR, when present in 100-fold excess could completely inhibit the formation of this constitutive VEGF RNA-protein complex.

A hypoxia-inducible RNA-protein complex was mapped by EMSA between VEGF 3′ UTR nucleotides 1412-1754 (GenBank Accession No. U22372). This complex thus maps to a site within a region of the VEGF mRNA 3′ UTR identified in the *in vitro*

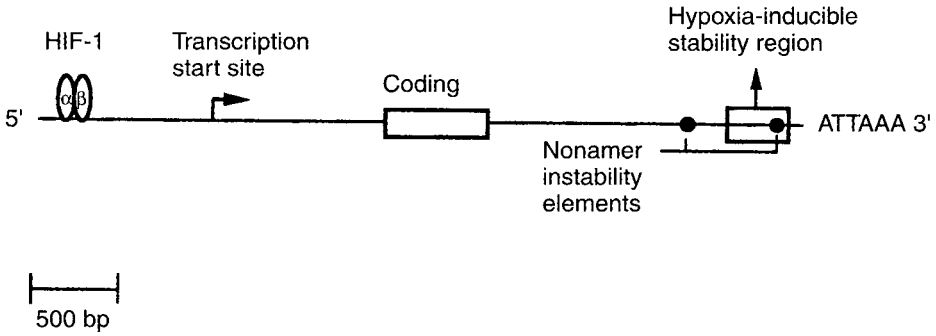

Fig. 1. *A simple schematic summary diagram of the regulation of steady-state VEGF mRNA by hypoxia.* (HIF-1) Hypoxia-inducible factor 1 mediates the transcriptional activation of VEGF by hypoxia. The transcription start site was defined by primer extension and RNase protection assay. (Coding) designates the open reading frame for the VEGF protein. The nonamer instability element, UUAUUUAUU, is a sequence motif that mediates the rapid turnover of multiple cytokine mRNA including VEGF mRNA. The Hypoxia-Inducible Stability Region is a 500 base fragment of RNA critical for the hypoxic stabilization of VEGF 3′ UTR transcripts in an *in vitro* degradation assay and which forms a hypoxia-inducible RNA-protein complex. The polyadenylation signal ATTAAA that is predominately used to generate the 3.7 kb VEGF mRNA is indicated. Three additional potential polyadenylation sites 5′ to this site are not shown.

RNA degradation assay to be critical for its preferential stabilization by S-100 extracts from hypoxic cells. This RNA-protein complex was induced approximately twofold by EMSA using hypoxic versus normoxic S-100 extracts. This complex was not effectively competed with a 500-fold excess of iron response element transcript or Epo 3′ UTR transcripts. Proteinase K treatment of the extracts completely inhibited formation of the complex [23].

Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein

Solid tumor growth is dependent upon tumor angiogenesis induced by angiogenic factors such as VEGF. This provides the tumor with nutrients and oxygen as well as an ability to establish metastasis via the circulatory system. Many tumor cell lines have been demonstrated to constitutively overexpress VEGF via constitutive stabilization of VEGF mRNA [22]. We have recently demonstrated that one mechanism for this constitutive stabilization of VEGF is via inactivation of the von Hippel-Lindau (VHL) tumor suppresser gene [28]. Inactivation of vHL protein has been implicated in the pathogenesis of renal carcinomas and central nervous system hemangioblastomas. These are highly vascular tumors that overproduce angiogenic peptides such as VEGF. Renal carcinoma cells lacking wild-type VHL were found to produce elevated levels of mRNAs encoding VEGF, the glucose transporter Glut-1, and platelet-derived growth factor B chain under both normoxic and hypoxic conditions. Reintroduction of the wild-type, but not mutant, VHL into these cells specifically inhibited the production of these mRNAs under normoxic conditions, thus restoring the hypoxia inducibility of these genes. Furthermore, the stability of VEGF mRNA was prolonged approximately fourfold in cells lacking wild-type VHL compared to cells in which wild-type VHL had been reintroduced. Transient transfection using the VEGF 5′ promoter driving expression of a luciferase reporter gene and nuclear run off studies failed to demonstrate any difference in the hypoxic regulation of VEGF in the mutant and wild type VHL cells. Thus, VHL appears to play a critical role in the transduction of signals generated by changes in ambient oxygen tension. Current studies are exploring potential interactions between the VHL protein and the hypoxia-inducible protein complex that binds to the VEGF mRNA 3′ UTR discussed above.

Summary

The regulation of VEGF production is mediated by both transcriptional and post-transcriptional mechanisms. A schematic model of elements involved in hypoxic regulation of VEGF is shown in Figure 1.

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References

1. Shweiki D, Itin A, Soffer D, Keshet E: Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* 359:843–845, 1992
2. Sabri MN, Disciascio G, Cowley MJ, Alpert D, Vetrovec GW: Coronary collateral recruitment: Functional significance and relation to rate of vessel closure. *Am Heart J* 121:876–80, 1991
3. Plate KH, Breier G, Weich HA, Risau W: Vascular endothelial growth factor is a potential tumor angiogenesis factor in human gliomas in vivo. *Nature* 359:845–848, 1992
4. Aiello LP, Avery RL, Arrigg PG, Keyt BA, Jampel HD, Shah ST, Pasquale LR, Thieme H, Iwamoto MA, Park JF, Nguyen HV, Aiello LM, Ferrara N, King G: Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. *N Engl J Med* 331:1480–1487, 1994
5. Levy AP, Tamargo R, Brem H, Nathans D: An endothelial cell growth factor from the mouse neuroblastoma cell line NB41. *Growth Factors* 2:9–19, 1989

6. LEUNG DW, CACHIANES G, KUANG WJ, GOEDDEL DV, FERRARA N: Vascular endothelial growth factor is a secreted angiogenic mitogen. *Science* 246:1306–1309, 1989
7. KIM KJ, LI B, WINER J, ARMANINI M, GILLET N, PHILLIPS HS, FERRARA N: Inhibition of VEGF-induced angiogenesis suppresses tumor growth in vivo. *Nature* 362:841–844, 1993
8. GOLDBERG MA, SCHNEIDER TJ: Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J Biol Chem* 269:4355–4359, 1994
9. LEVY AP, LEVY NS, LOSCALZO J, CALDERONE A, TAKAHASHI N, YEO K-T, KOREN G, COLUCCI WS, GOLDBERG MA: Regulation of vascular endothelial growth factor in cardiac myocytes. *Circ Res* 76:758–766, 1995
10. LEVY AP, LEVY NS, WEGNER S, GOLDBERG MA: Transcriptional regulation of the rat vascular endothelial growth factor gene by hypoxia. *J Biol Chem* 270:13333–13340, 1995
11. GOLDBERG MA, DUNNING SP, BUNN HF: Regulation of the erythropoietin gene: Evidence that the oxygen sensor is a heme protein. *Science* 242:1412–1415, 1988
12. CLAFFEY KP, WILKISON WO, SPIEGELMAN BM: Vascular endothelial growth factor: Regulation by cell differentiation and activated second messenger pathways. *J Biol Chem* 267:16317–16322, 1992
13. STEIN I, NEEMAN M, SHWEIKI D, ITIN A, KESHET E: Stabilization of vascular endothelial growth factor mRNA by hypoxia and hypoglycemia and coregulation with other ischemia-induced genes. *Mol Cell Biol* 15:5363–5368, 1995
14. WANG GL, JIANG BH, RUE EA, SEMENZA GL: Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O_2 tension. *Proc Natl Acad Sci USA* 92:5510–5514, 1995
15. WU X, DISCHER D, BISHOPRIC N, WEBSTER KA: Zinc fingers are redox sensors during acute oxidative stress. (abstract) *Circ* 90:1343A, 1994
16. ABATE C, LUK D, CURRAN T: Rapid regulation of fos and jun DNA-binding activity in vitro. *Science* 249:1157–1161, 1990
17. FIRTH J, EBERT B, RATCLIFFE P: Hypoxic regulation of lactate dehydrogenase A. *J Biol Chem* 270:21021–21027, 1995
18. SEMENZA GL, WANG GL: A nuclear factor induced by hypoxia via de novo protein wynthesis binds to the human erythropoictin gene enhancer at a site required for transcriptional activation. *Mol Cell Biol* 12:5447–5454, 1992
19. MADAN A, CURTIN PT: A 24-base pair sequence 3′ to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer. *Proc Natl Acad Sci USA* 90:3928–3932, 1993
20. BLANCHARD KL, ACQUAVIVA AM, GALSON DL, BUNN HF: Hypoxic induction of the human erythropoietin gene: Cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. *Mol Cell Biol* 12:5373–5385, 1992
21. SHIMA DT, DEUTSCH U, D'AMORE PA: Hypoxic induction of vascular endothelial growth factor (VEGF) in human epithelial cells is mediated by increases in mRNA stability. *FEBS Lett* 370:203–208, 1995
22. WHITE FC, CARROLL SM, KAMPS MP: VEGF mRNA is reversibly stabilized by hypoxia and persistently stabilized in VEGF-overexpressing human tumor cell lines. *Growth Factors* 12:289–301, 1995
23. LEVY AP, LEVY NS, GOLDBERG MA: Post-transcriptional regulation of rascular endothelial growth factor by hypoxia. *J Biol Chem* 271: 2746–2753, 1996
24. SHAW G, KAMEN R: A conserved AU sequence from the 3′ untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell* 46:659–667, 1986
25. LAGNADO CA, BROWN CY, GOODALL GJ: AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: The functional sequence within AU-rich elements may be UUAUUUA(A/U) (A/U). *Mol Cell Biol* 14:7984–7995, 1994
26. ZUBIAGA AM, BELASCO JG, GREENBERG ME: The nonamer UUAUUUAUU is the key AU-rich sequence motif that mediates mRNA degradation. *Mol Cell Biol* 15:2219–2230, 1995
27. CZYZYK-KRZESKA MF, DOMINSKI Z, KOLE R, MILLHORN DE: Hypoxia stimulates binding of a cytoplasic protein to a pyrimidine-rich sequence in the 3′-untranslated region of rat tyrosine hydroxylase mRNA. *J Biol Chem* 269:9940–9945, 1994
28. ILIOPOULOS O, LEVY AP, JIANG C, KAELIN WG, GOLDBERG MA: Negative regulation of hypoxia-inducible genes by the von Hippel-Lindau protein. *Proc Natl Acad Sci USA* 93:10595–10599, 1996